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2',3'-seco PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES, INCLUDING STRUCTURAL ANALOGUES OF 3':5'-CYCLIC CMP AND UMP, AND THEIR BEHAVIOUR IN SEVERAL ENZYME SYSTEMS

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SUMMARY: Cyclization of 2',3'-seco-5'- CMP and UMP with dicyclohexylcarbodiimide leads to 2',3'-seco-3':5'- cCMP and cUMP, formal structural analogues of 3':5'- cCMP and cUMP. $POCl_3$ phosphorylation of 2',3'-secocytidine gave the same product in 50% yield, plus three additional seco nucleotides, one of which was independently obtained by enzymatic phosphorylation with the wheat shoot phosphotransferase system. The behaviour of these nucleotides has been examined in several enzyme systems. In particular, the seco 3':5'-cyclic phosphates are resistant to beef heart cyclic nucleotide phosphodiesterase, but are slowly hydrolyzed to the monophosphates by higher plant cyclic nucleotide phosphodiesterase.

Oxidation of ribonucleosides (or their 5'-phosphates) with periodate, followed by reduction with borohydride, leads to cleavage of the C(2')-C(3') bond to give 2',3'-seco nucleosides (or their 5'-phosphates) (1).

Our interest in such compounds stems from their formal structural resemblance to acyclonucleoside antiviral agents, acycloG and DHPG (Scheme 1), the activities of which are mediated by their intracellular phosphorylation, initially by viral thymidine kinase (2). From Scheme 1 it will be seen that, if the 5'-phosphate and 3'-OH of a seco nucleotide are suitably oriented, it should be feasible to prepare the corresponding 3':5'-cyclic phosphates, structural analogues of the biologically important cAMP, cGMP, cCMP, etc. (Scheme 2). We have, in fact, succeeded in preparing such analogues of cAMP

ABBREVIATIONS: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; TEA, triethylammonium; PDase, phosphodiesterase; cPDase, cyclic nucleotide phosphodiesterase; acycloG or acyclovir, 9-(2-hydroxyethoxymethyl)guanine; DHPG, 9-(1,3-dihydroxypropoxymethyl)guanine; TLC, thin-layer chromatography.

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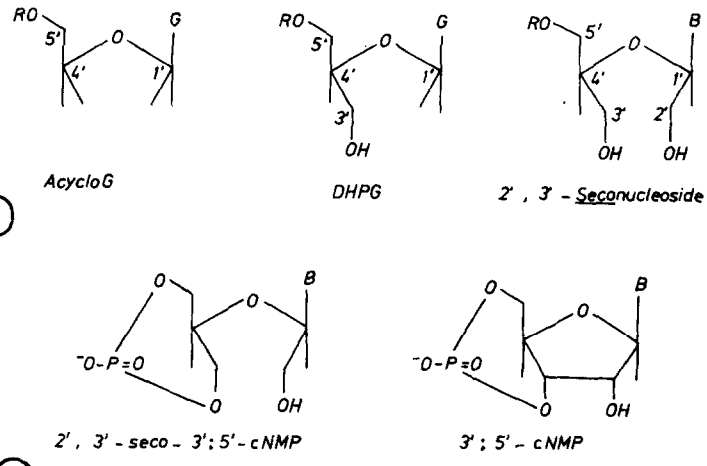

Scheme 1. Acyclonucleosides (R = H) and acyclonucleotides (R = $PO(OH)_2$). G = guanine; B = any base. Note: The numbering system for the carbon atoms of the acyclo chains is similar to that for the carbons of the pentose ring.

Scheme 2. N = nucleoside or acyclonucleoside; B = base.

and related congeners (Stolarski et al., in preparation). During the course of this work, Tolman et al. (3) reported the preparation of the cyclic phosphate of DHPG, a potent broad-spectrum *in vitro* antiviral agent. Bearing in mind the probable important role of the conformations of acyclonucleosides (4), and their antimetabolic activities, which are dependent on their intracellular phosphorylation (2), we here examine the behaviour of *seco* nucleotides of uridine and cytidine in a number of enzymatic systems.

MATERIALS AND METHODS

Materials. Nucleosides and nucleotides were from Sigma (St. Louis, MO.) and Calbiochem (Lucerne, Switzerland). Pyridine was distilled over CaH_2, and DMF was purified by azeotropic distillation with water and benzene, then distilled under reduced pressure. Dowex resins (200/400 mesh) were from BioRad (Richmond, VA.), DEAE-Sephadex A-25 from Pharmacia (Uppsala, Sweden), Merck (Darmstadt, GFR) cellulose F-254 plates were used for TLC with solvents A-D, and silica gel 60F-254 plates with solvent E (see Table 1).

Enzymes. Snake venom 5'-nucleotidase (EC 3.1.3.5), beef heart cPDase (EC 3.1.4.17) and rye grass 3'-nucleotidase (EC 3.1.3.6) were from Sigma. Potato tuber cPDase (EC 3.1.4.-) was a highly purified preparation elsewhere described (5). Purified *E. coli* uridine phosphorylase (EC 2.4.2.3) was prepared according to Vita & Magni (6), and cytidine deaminase (EC 3.5.4.5) was a crude extract from the same source totally free of uridine phosphorylase. *E. coli* alkaline phosphatase was a product of Worthington (Freehold, N.J.).

SYNTHETIC METHODS

2',3'-seco uridine and cytidine were prepared essentially as described by Lerner (7), and desalted by elution with 2.5% NH_4OH from a column of

Dowex 50Wx8 (H^+). Secocytidine was obtained in crystalline form from anhydrous ethanol in 56% yield, m.p. 167-169°C. Secouridine was obtained in 60% yield, chromatographically homogeneous, but could not be crystallized.

2',3'-Seco-5'-CMP. To 3.23 g (10 mmol) 5'-CMP in 60 ml water, brought to neutrality with triethylamine, was added 2.72 g (12 mmol) $HIO_4 \cdot 2H_2O$. The solution was stirred for 1 hr, with addition of triethylamine to maintain neutral pH. The reaction was terminated by addition of 1 ml ethylene glycol, followed by portionwise addition, with stirring for 3 hr of 1.22 g (32 mmol) $NaBH_4$. Stirring was continued for 3 hr, and the solution diluted to 300 ml and loaded on a 3.5x21 cm column of Dowex 1x4($HCOO^-$). On elution with a linear gradient of H_2O-1 M HCOOH (2 l), the product appeared at about 0.35 M HCOOH. The pooled fractions were brought to dryness under reduced pressure, the residue taken up in a small volume of water, and the product precipitated with acetone to yield 2.91 g (89%) as the free acid, m.p. 208-210°C, chromatographically homogeneous. Treatment with alkaline phosphatase led to quantitative conversion to secocytidine.

2',3'-Seco-5'-UMP. Prepared from 2.2 g 5'-UMP·Na_2 essentially as for 2',3'-seco-5'-CMP, above, but with elution from the column with a linear gradient of H_2O-5 M HCOOH (4 l). The product eluted at about 3 M HCOOH, and was converted to the Na_2 salt by passage through a column of Dowex 50x8(Na^+). The effluent was concentrated to small volume, and anhydrous ethanol added to precipitate 1.72 g (78%) of a white powder, m.p. 162-166°C, chromatographically homogeneous, and quantitatively converted to secouridine with alkaline phosphatase.

2',3'-Seco-3':5'-cCMP. 2',3'-seco-5'-CMP (390 mg, 1.2 mmol) was converted to the 4-morpholine-N,N'-dicyclohexylcarboxamidine salt, which was dried by evaporation from anhydrous pyridine and dissolved in 40 ml anhydrous DMF. This solution was added dropwise to a boiling solution of 740 mg (3.6 mmol) DCC in 110 ml anhydrous pyridine. The solution was refluxed for a further 1.5 hr, and the reaction terminated by addition of 5 ml water. The mixture was brought to dryness, and the residue brought to dryness several times from water to remove residual pyridine, then taken up in 200 ml water and filtered through a Celite pad and loaded on a 3.5x28 cm column of Dowex 1x8($HCOO^-$). On elution with a linear gradient of H_2O-1 M HCOOH (3 l), the product appeared at about 0.32 M. The pooled fractions were brought to dryness, and the residue taken up in a small volume of water and precipitated with absolute ethanol, to yield 310 mg (84%) of a white powder, m.p. 208-213°C (dec.), chromatographically homogeneous.

2',3'-seco-3':5'-cUMP. Prepared from 405 mg (1.25 mmol) of 2',3'-seco-5'-UMP essentially as in the preceding paragraph, but with isolation of the product on a 2.5x46 cm column of DEAE-Sephadex A-25(HCO_3^-). Elution was with a linear gradient of H_2O-0.5 M TEA bicarbonate (2 l). The pooled fractions of the product (at ~0.18 M) were brought to dryness, and the residue brought to dryness several times from ethanol to remove residual TEA bicarbonate. The product was converted to the Na^+ salt by passage through a column of Dowex 50Wx8(NA^+), the effluent reduced to small volume, and the product precipitated with absolute ethanol to yield 375 mg (70%) of a white powder, m.p. 210-215°C, chromatographically homogeneous.

Phosphorylation of 2',3'-secocytidine. Secocytidine (240 mg, 1 mmol) was phosphorylated with $POCl_3$ as described by Yoshikawa et al. (8). Following neutralization of the reaction mixture with $NaHCO_3$, it was loaded on a 3.5x22 cm column of DEAE-Sephadex A-25(HCO_3^-). A water wash released 7% non-reacted nucleoside. The products were then eluted with a linear gradient of H_2O-1 M TEA bicarbonate (4 l), with collection of 19-ml fractions. The first peak, identified as 2',3'-seco-3':5'-cCMP (fractions 28-42, 53% yield), was followed by a second, presumably an enantiomeric mixture of 2',3'-seco-5'(3')-CMP (fractions 52-65, 13%). A third peak, 2',3'-seco-3':5'-cCMP additionally phosphorylated at 2' (fractions 78-91, 11%), was succeeded by a fourth, 2',3'-secocytidine-3',5'-bismonophosphate (fractions 93-110, 14%).

2',3'-seco-2'-CMP. To a stirred suspension of 140 mg (0.5 mmol) of 2',3'-seco-3',5'-O-isopropylidenecytidine (prepared as for isopropylidene-

cytidine, ref. 9) in 8 ml $(EtO)_3PO$ at 0°C, was added 200 µl (2.2 mmol) $POCl_3$ and stirring continued until a clear solution formed. The mixture was left at 4°C for 60 hr, 5 ml water added and, after 15 min, 20 ml 50% formic acid, and the mixture left at room temp. for 3 hr. Formic acid and water were removed under reduced pressure, 25 ml water added, the mixture neutralized with NH_4OH, and loaded on a 2x20 cm column of Dowex 1x8($HCOO^-$). Elution with water released non-reacted secocytidine, followed by the nucleotide. The pooled nucleotide fractions were freed of formic acid under reduced pressure, the residue taken up in water, and deposited on a 2x22 cm column of DEAE-Sephadex A-25(HCO_3^-). A linear gradient of H_2O-0.4 M TEA bicarbonate eluted the product at about 0.21 M. The pooled fractions were brought to dryness several times from water and ethanol to remove TEA bicarbonate, the nucleotide converted to the Na_2 salt on Dowex 50Wx8(Na^+), and precipitated from a 0.5 ml solution with anhydrous ethanol to yield 87 mg (47%) of a white, chromatographically homogeneous powder. It was quantitatively converted to secocytidine by alkaline phosphatase.

Enzymatic phosphorylation of 2',3'-secocytidine. A 120 mg (0.5 mmol) sample of 2',3'-secocytidine was phosphorylated with the wheat shoot phosphotransferase system (10) and deposited on a 2x18 cm column of Dowex 1x8 ($HCOO^-$). A water wash released 92% nonreacted nucleoside. Elution with a linear gradient of H_2O-1 M HCOOH (1 l), with collection of 19-ml fractions, led to one peak (fractions 14-16, 5%), shown below to be an enantiomeric mixture of 2',3'-seco-5'(3')-CMP, followed by a second (fractions 17-19, 2.5%), identified as 2',3'-seco-2'-CMP by TLC (Table 1) and by its identity with the product of chemical phosphorylation (above). Each product was quantitatively converted to secocytidine by alkaline phosphatase.

RESULTS

As described above, syntheses of 2',3'-secouridine, cytidine, 5'-UMP and 5'-CMP, by standard procedures (7), combined with column chromatography, proceeded in high yield to furnish chromatographically homogeneous products (see Table 1). While only secocytidine could be crystallized, the other products exhibited reasonably good melting points further testifying to their purity.

As expected, cyclization of 2',3'-seco 5'-CMP and 5'-UMP with DCC (11) also led to good yields of the 3',5'-cyclic phosphates, again chromatographically homogeneous, and with well-defined melting points.

Particularly interesting was the $POCl_3$ phosphorylation (8) of secocytidine. In the case of the parent nucleosides this normally leads to formation of the 5'-phosphates. With secocytidine, the principal product was the 3':5'-cyclic phosphate (53% yield), identical with that obtained by cyclization of seco-5'-CMP. We have eslewhere found that $POCl_3$ phosphorylation of secoadenosine gave the 3':5'-cyclic phosphate as the major product (50%), additionally confirmed by NMR spectroscopy (Stolarski et al., in preparat-

Table 1. R_f values of 2',3'-seco analogues with solvent systems (v/v): (A) isopropanol:water:25% NH_4OH (4:4:1); (B) isopropanol:water:acetic acid (4:4:1); (C) isopropanol:water (10:4); (D) isopropanol:1% ammonium sulphate (7:3); (E) chloroform:methanol (8:2). A dash indicates tailing.

Compound	Solvent system				
	A	B	C	D	E
2',3'-Secocytidine	0.83	0.79	0.57	0.74	0.07
2',3'-Secouridine	0.77	0.77	0.67	0.66	0.27
5'-UMP	0.63	0.62	0.25	0.70	0.00
2',3'-Seco-5'(3')-UMP	0.70	0.70	0.34	0.82	0.00
2',3'-Seco-3':5'-cUMP	0.77	0.70	0.39	0.62	0.00
5'-CMP	0.65	0.53	0.14	0.72	0.00
2',3'-Seco-5'(3')-CMP	0.73	0.58	0.22	0.84	0.00
2',3'-Seco-3':5'-cCMP	0.80	0.63	0.27	0.69	0.00
2',3'-Secocytidine-3',5'-bis(monophosphate)	0.45	0.23	-	-	0.00
2',3'-Seco-3':5'-cCMP-2'-monophosphate	0.59	0.40	-	-	0.00
2',3'-Seco-3',5'-O-isopropylidenecytidine	0.85	0.86	0.75	0.74	0.33
2',3'-Seco-2'-CMP	0.75	0.52	-	-	0.00

ion). This recalls various reported instances of formation of 3':5'-cyclic phosphates of other nucleosides by phosphorylation with $POCl_3$ and other phosphorylating agents (12). By contrast, $POCl_3$ treatment of DHPG gave, as the principal product, the 3',5'-bis(monophosphate), and only 7% of the cyclic phosphate (3).

The other products of $POCl_3$ phosphorylation of secocytidine were identified as follows: The product from peak II migrated on the column, and on TLC, as 2',3'-seco-5'-CMP, and was quantitatively converted to secocytidine with alkaline phosphatase; however, this does not exclude the possibility that it may be an enantiomeric mixture of 3'- and 5'- phosphates, not resolved on TLC. The product from peak III was readily identified since, on treatment with alkaline phosphatase, it was quantitatively converted to seco-3':5'-cCMP, hence must be seco-3':5'-cCMP additionally phosphorylated at 2'. The product from peak IV migrated on the column, and on TLC, like a bis(monophosphate) or diphosphate, was converted by alkaline phosphatase, via an intermediate migrating on TLC like seco-5'-CMP, to secocytidine and therefore must be secocytidine-3',5'-bis(monophosphate); 2',3'- or 2',5'-bis(monophosphate) are excluded, since then one of the intermediate(s) during alkaline

phosphatase hydrolysis would have been the 2'-monophosphate, readily distinguished from 3'(5')-monophosphates on TLC with solvent B (see Table 1). The foregoing results are similar to those obtained with $POCl_3$ phosphorylation of secoadenosine, confirmed by ^1H and ^{13}C NMR (Stolarski et al., in preparation).

Enzymatic trials. Using standard spectrophotometric assay procedures, as well as TLC (see Table 1), it was found that secocytidine is neither a substrate, nor an inhibitor, of cytidine deaminase. Secouridine was not a substrate of uridine phosphorylase; and, under conditions where the uracil analogue of acycloG inhibited the enzyme with a K_i of 15 μM (13), and the uracil analogue of DHPG even more effectively (A. Drabikowska et al., in preparation), the seco analogue did not detectably inhibit. Under conditions where 5'-CMP was rapidly dephosphorylated by 5'-nucleotidase, neither seco-5'-, nor 2'-, CMP was a substrate or inhibitor.

Particularly interesting were results with 3'-nucleotidase. With standard assay conditions (14), using TLC (Table 1), secocytidine-2'- and 5'-phosphates were not substrates, However secocytidine-3',5'-diphosphate was quantitatively converted, at about half the rate for 3'-CMP, to a mononucleotide which must be seco-5'-CMP, a finding consistent with the fact that this enzyme hydrolyzes ribonucleoside-3',5'-diphosphates to the 5'-phosphates (14).

Susceptibility of seco-3':5'-cCMP to beef heart cPDase was examined both by TLC, and by alkaline phosphatase liberation of P_i following opening of the cyclic phosphate ring (5). No detectable hydrolysis was noted under conditions where cCMP and cAMP were readily hydrolyzed. By contrast the secocytidine cyclic phosphate (as well as seco-3':5'-cAMP), was readily hydrolyzed to the mononucleotide by potato tuber cPDase, at a rate about 10% that for cAMP.

DISCUSSION

The resistance of seco-3':5'-cCMP to beef heart cPDase is of interest in relation to its susceptibility to higher plant cPDase. However, mammalian cells contain a non-conventional so-called cCMP PDase (15) with properties similar in some respects to those of higher plant cPDase (5). This prompted

us to conduct a preliminary screening of several mammalian cell lines for such activity, and we have found that, in fact, a sonicated extract of Ehrlich carcinoma cells hydrolyzes seco-3':5'-cCMP and cAMP at rates about 15% of that for 3':5'-cAMP. This is presently being further investigated.

The susceptibility of secocytidine-3',5'-diphosphate to 3'-nucleotidase is relevant to the specificity of this enzyme, which hydrolyzes ribonucleoside-3',5'-diphosphates to the 5'-phosphates, but is inactive vs arabinonucleoside-3',5'-diphosphates and 2'-deoxyribonucleoside-3',5'-diphosphates (14). It follows that binding of the seco analogue by the enzyme must be accompanied by suitable orientation of the 2'-OH. In particular, 2',3'-seco-2'-deoxycytidine-3',5'-diphosphate should not be a substrate, and synthesis of this compound is under way.

We have found that phosphorylation of compounds like DHPG with wheat shoot nucleoside phosphotransferase proceeds relatively efficiently ($\sim$ 50%). It is therefore surprising that secocytidine, which possesses three primary hydroxyls, is phosphorylated to the extent of only 7.5%. Since for both types of compounds, the only products of enzymatic phosphorylation are the monophosphates, it appears that in the seco analogue there may be mutual steric hindrance between the 5'(3') and the 2' OH groups.

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REFERENCES

1. Lerner, L.M. and Rossi, R.R. (1972) Biochemistry 11, 1181-1188.
2. Elion, G.B. (1982) Am. J. Med. 73, 7-13.
3. Tolman, R.L., Field, A.K., Karkas, J.D., Wagner, A.F., Germershausen, Crumpacker, C. and Scolnick, E.M. (1985) Biochem. Biophys. Res. Commun. 128, 1329-1335.
4. Birnbaum, G.I., Stolarski, R., Kazimierczuk, Z. and Shugar, D. (1985) Can. J. Chem. 63, 1215-1221.
5. Zan-Kowalczewska, M., Bartkiewicz, M., Sierakowska, H. and Shugar, D. (1984) Biochim. Biophys. Acta 788, 62-73.
6. Vita, A. and Magni, G. (1983) Anal. Biochem. 133, 153-157.
7. Lerner, L.M. (1970) Carbohyd. Res. 13, 465-469.
8. Yoshikawa, M., Kato, T. and Takenishi, T. (1969) Bull. Chem. Soc. Japan 42, 3505-3508.
9. Hampton, A. (1961) J. Am. Chem. Soc. 83, 3640-3648.

10. Giziewicz, J. and Shugar, D. (1978) in "Nucleic Acid Chemistry" (L.B. Townsend and R.S. Tipson, eds.) 2, 955-961; (1975) Acta Biochim. Polon. 22, 87-98.
11. Smith, M., Drummond, G.I. and Khorana, H.G. (1961) J. Am. Chem. Soc. 83, 698-706.
12. Van der Meer, S. and Pouwells, H. (1969) J. Med. Chem. 12, 534-535.
13. Niedzwicki, J.G., el Kouni, M.H., Chu, H.S. and Cha, S. (1984) Biochem. Pharmacol. 30, 2097-2101.
14. Barker, G.R. and Lund, G. (1962) Biochim. Biophys. Acta 55, 987-988.
15. Helfman, D.M. and Kuo, J.F. (1982) J. Biol. Chem. 257, 1044-1047.